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Short Communication

The role of cell differentiation state and HMG-I/Y in the expression of transgenes flanked by matrix attachment regions

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Abstract

The tobacco nuclear matrix attachment region (MAR), RB7, has been shown to have a much greater effect on transgene expression in cultured cells than in transgenic plants. This is comparable to work in mouse systems showing that MARs have a positive effect on transgene expression in embryonic tissues but not adult tissues. There are several possible explanations for these observations. One is that cell differentiation state and proliferation rate can affect MAR function. We tested this possibility by initiating suspension cell cultures from well-characterized transgenic plants transformed with *35S::GUS* with and without flanking MARs and then comparing GUS specific activity in the cell lines to those of the transgenic plants from which the cell lines were derived. If cell differentiation state and proliferation rate do affect MAR function, we would expect the ratio of transgene expression (cell suspensions : plants) to be greater in MAR lines than in control lines. This turned out not to be the case. Thus, it appears that MAR function is not enhanced simply because cells in culture divide rapidly and are not differentiated. Because in animal systems the chromosomal protein HMG-I/Y has been shown to be upregulated in proliferating cells and may have a role in MAR function, we have also examined the levels of the tobacco HMG-I/Y homolog by immunoblotting. The level of this protein does not differ between primary transformant cultured cells (NT-1) and *Nicotiana tabacum* plants (SR-1). However, a higher molecular weight cross-reacting polypeptide was found in nuclei from the NT-1 cell suspensions but was not detected in SR-1 leaf nuclei or cell suspensions derived from the SR-1 plants.

Introduction

Nuclear matrix attachment regions (MARs) are DNA sequences that bind to isolated nuclear matrices (reviewed in Spiker & Thompson, 1996). These regions are generally AT-rich (70%) and typically around 1 kb in size. MARs have been shown to be important for the proper regulation of endogenous genes. For example, in transgenic mice, an Ig mu gene lacking its endogenous MAR is up to 1000-fold less active in splenic B cells compared to the wild-type Ig mu gene (Forrester et al., 1994). Several other MARs have been shown to have positive effects on the expression of transgenes (reviewed by Allen et al., 2000).

The enhancement of transgene expression conferred by MARs has been associated with cell differentiation state. For example, Thompson and colleagues (1994) noted that flanking MARs from the human β-interferon gene increased the expression of a mur-

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ine *HSP70.1::LUC* reporter gene in mouse embryos but not newborn mice. They also reported a strong correlation between HMG-I/Y transcript levels and MAR-associated transgene expression during the development of the preimplantation embryo from the two-cell through blastocyst stages (Thompson et al., 1995). The authors have cited the 'chromatin opening' model (Käs et al., 1993; Zhao et al., 1993) to explain their observations. In brief, Laemmli and colleagues (Izaurralde et al., 1989) have demonstrated that MARs can serve as sites of nucleation for the cooperative assembly of linker histones (e.g. histone H1) along the MAR and into flanking non-MAR DNA. This assembly of linker histones may cause chromatin condensation and thus establish a domain of inactive chromatin at the MAR. According to the model, in the presence of AT-binding proteins, such as HMG I/Y-type proteins, the binding of linker histones to MARs is destabilized, thus disrupting the associated higher order structure and establishing a transcriptionally active domain.

In our work with plants, we have also observed a correlation between differentiation state and the level of transgene expression stimulated by MARs. When cultured cells (tobacco NT-1) were transformed using microprojectile bombardment, the RB7 MAR (Hall et al., 1991) was found to increase average expression levels of a *GUS* transgene 60-fold relative to controls (Allen et al., 1996). However, when the same constructs were introduced into tobacco plants, we observed only a three-fold stimulation of expression (Ülker et al., 1999). This moderate RB7-associated increase has also been observed in poplar, rice and pine plants (Han et al., 1997; Vain et al., 1999; Levée et al., 1999).

Mammalian HMG I/Y proteins have been shown to be present at elevated levels in rapidly dividing, undifferentiated cells (Goodwin et al., 1985; Johnson et al., 1990). In plants, RNA analyses revealed some preferential expression of the *Arabidopsis* HMG I/Y transcripts in non-terminally differentiated organs, although the genes were expressed in all plant tissues (Gupta et al., 1997; Ascenzi, unpublished observations). In addition, genes for two rice proteins containing the AT-hook motif of HMG-I/Y are preferentially expressed in embryonic cell-types (Meijer et al., 1996). Therefore, these chromosomal proteins may be important constituents of chromatin in undifferentiated plant cells.

In this communication, we investigate the relationship between cell differentiation, abundance of HMG-I/Y homologs, and MAR function in plants. We have generated cell culture lines from transgenic plants transformed with and without MARs flanking reporter transgenes. We find that the conversion of differentiated cells in tobacco leaves to suspension cells does not change the magnitude of MAR-enhanced transgene expression. Furthermore, the plant HMG-I/Y-like protein is not more abundant in cell culture than in differentiated cells.

Experimental findings

Our strategy for examining the role of cell differentiation on MAR-enhanced transgene expression was to obtain cell lines from transformed plant lines. If differentiation state is responsible for stimulating MAR-associated transgene expression in NT-1 cells to a greater extent than in SR-1 plants, one would expect the ratio of transgene expression levels (cell suspensions : leaf tissue) to be greater in the MAR lines than the control lines.

We generated 10–12 callus cultures from each of seven independently transformed doubled-haploid SR-1 plant lines (Ülker et al., 1999) (see Table 1 for methods). Of these calli, 18 (up to four for each plant line) were established as suspension cultures (SR-1 cell lines). Samples were taken at mid-log phase from established cultures (approximately four months after initial transfer to liquid) and assayed for GUS activity. Mean GUS specific activities from three to four replicates were calculated for each culture. These means were then averaged with other cultures independently derived from the same transgenic plant line (Table 1). The variation among cell lines derived from the same plant line was minimal (Table 1 and data not shown). The average specific activities in each group of cell lines were then compared to activities obtained from mature leaves of the original plant lines. The activities measured in leaf extracts at this developmental stage did not differ greatly from extracts of younger tissues (data not shown).

In the control line set, C83 and C91 (without MARs), the average GUS activities derived from cell suspensions were 2.8–3.5 greater than activities from leaf extracts (Table 1). The corresponding MAR-line ratios range from 0.15 to 20. Although there was some variation in the cell suspension to leaf tissue ratios, the overall results cannot account for the differences between the 60-fold MAR effect found when the originally-transformed cells were in suspension

Table 1. Comparison of GUS specific activities in leaves and in cell suspensions

Line[a]	Sp. Act., Plant[b]	Sp. Act., Cells (n)[c]	Cells/Plant[d]
C83	350	980 (4)	2.8 ± 0.52
C91	450	1600 (3)	3.5 ± 0.27
M38	370	2400 (4)	6.6 ± 2.6
M36	340	240 (2)	0.71 ± 08
M117	460	1100 (1)	2.3
M80	50	970 (3)	20 ± 6.7
M106	180	30 (1)	0.15

To initiate cultures, seedlings were germinated on GM medium (Valvekens et al., 1988). After eight days, explants derived from the hypocotyl or cotyledon were transferred to RMNO medium (Maliga et al., 1976) made with Murashige Minimal Organics Medium (Life Technologies Rockville, MD). Suspensions (5 ml) were started from well-established friable calli after three weeks of growth. Cultures (50 ml) were maintained in RMNO medium for two months before switching to a medium containing 2 mg/l 2,4-D.

[a]In the line nomenclature, the prefix ‘C’ indicates that the plant was transformed with pGHNC12 (no MARs) and ‘M’ indicates that the plant was transformed with pGHNC11 (containing MARs) (details of plasmids are found in Allen et al., 1996).

[b]Mean specific activities obtained from three replicate leaf samples obtained from 40-day-old plants harvested as described in Ülker et al. (1999). GUS activities were obtained by a luminometric assay, using the Glucuron substrate (Tropix, Bedford, MA). Specific activity is defined as ‘Fishman’ units per gram of tissue, where one unit is the amount of enzyme that will liberate 1.0 μg of phenolpthalein per hour at 37°C.

[c]Average of mean specific activities of n separate cultures derived from single plant line.

[d]Mean ratio of sp. act. in extracts derived from each cell suspension to leaf tissue sp. act. The standard errors of these values obtained are given.

culture (NT-1) and the three-fold effect found when the originally-transformed cells were in differentiated plant tissues (SR-1).

To evaluate whether the observed expression differences could be explained by differences in proliferation, the growth rates of the well-established NT-1 cell line and the recently established SR-1 cell lines were compared by measuring the packed cell volume. For this experiment, all cells were grown on the medium described in Allen et al. (1996). While the same final density was achieved in both types of cells, the mean growth rate of the SR-1 lines was approximately half that of NT-1 (data not shown). This result shows that our SR-1 cell lines do not achieve the same rate of growth as the established NT-1 cell line.

In order to investigate the role that HMG-I/Y abundance might play in the magnitude of MAR-enhanced transgene expression, we compared the levels of the tobacco HMG-I/Y homolog in intact plant leaves and cells in culture. Nuclei were isolated from both of these tissues. To assess the relative abundance of HMG-I/Y-type proteins within the two tobacco tissues, proteins from solubilized nuclei (equalized for DNA content) were analyzed by SDS-PAGE. Bands corresponding to the histones were seen in both tissue types in equal abundance (Figure 1A). Wheat germ HMGa has been shown to share homology to HMG-I/Y-type proteins as well as histone H1 (Arwood & Spiker, 1990; Grasser, 1995). An antiserum raised against wheat HMGa (Spiker & Everett, 1987) was used in western blots. These analyses revealed a strongly cross-reacting band of approximately 30 kDa in each of the lanes (Figure 1B). Because this protein, which clearly cross reacts with the HMGa antiserum, has mobility consistent with that expected for plant HMG-I/Y, and fits the operational definition of HMG proteins (in that it is extracted from nuclei by 2% trichloroacetic acid) (Spiker, 1984), we conclude that this protein is the tobacco HMG-I/Y homolog. This 30-kDa protein appears to be present in approximately equal abundance within the two tissue types, or perhaps even greater in the leaf tissue (Figure 1 and data not shown). The level of HMG-I/Y was also compared between NT-1 cells and a cell line derived from SR-1. We did not observe any difference between the two cell lines (data not shown). Therefore, we conclude, the abundance of the HMG-I/Y homolog is not correlated with differences in cell proliferation or MAR-mediated transgene expression levels in tobacco.

Interestingly, the western revealed another protein of approximate molecular mass of 80 kDa, which cross reacts with the HMGa antisera. This protein was found only in the nuclei of tobacco NT-1 cells (a suspension culture that has been maintained for many years) and not in tobacco leaves or in SR-1 suspension cultures (data not shown). Furthermore, a similarly sized HMG-I/Y-related protein was also observed in the rapidly proliferating BMS maize cell line (data not shown). In light of its high molecular mass and the fact that it does not conform to the operational definition of HMG proteins (extracted by 2% trichloroacetic acid) (data not shown), the 80 kDa cross reacting polypeptide cannot be considered an HMG. Non-HMG proteins that contain the AT-hook DNA-binding domain of HMG I/Y have been identified (Tjaden & Coruzzi, 1994; Meijer et al., 1996; Hofmann et al., 2000). The 80-kDa tobacco protein identified by the HMGa antiserum may be related to one of these proteins.

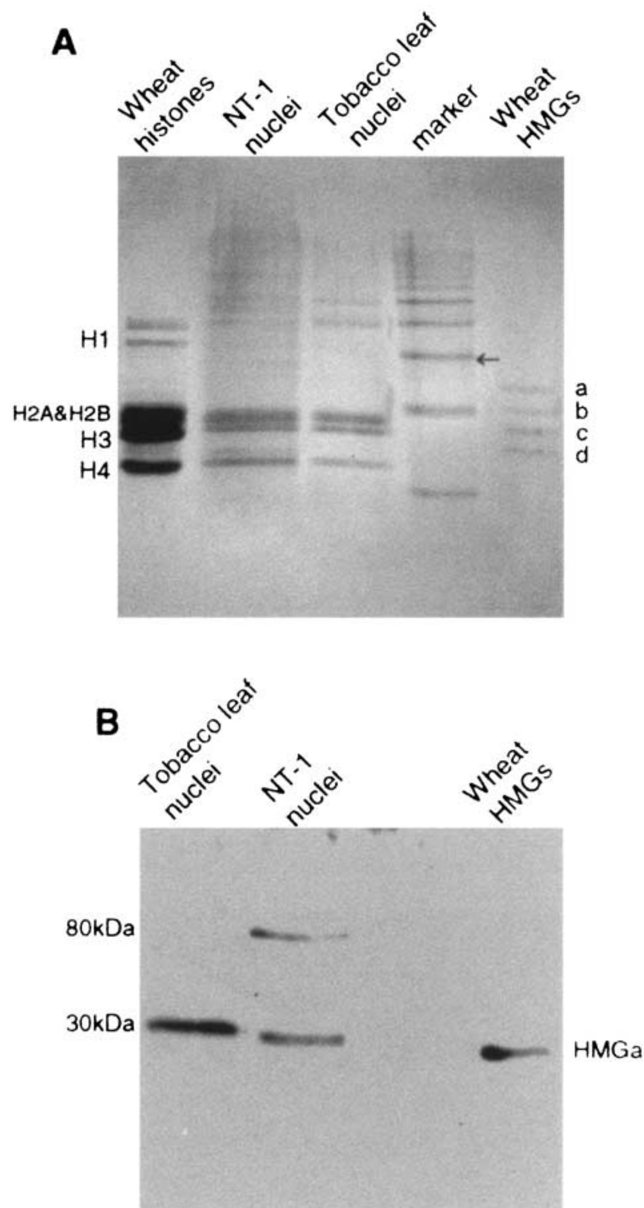

Figure 1. Analysis of tobacco HMG-I/Y homolog levels in nuclei isolated from cell suspensions and leaf tissue. Nuclei were isolated from 100 ml of 4-day-old mid-log phase cultures as described in Hall et al. (1991). Tobacco leaf nuclei were similarly isolated from leaves of 30-day-old seedlings. (A) SDS-PAGE of solubilized NT-1 and tobacco leaf nuclei. Equal aliquots of nuclei based on DNA content from NT-1 suspension cells and tobacco leaf tissue were solubilized in 2% SDS and applied to 18% SDS-polyacrylamide gels along with purified wheat germ histones (Spiker, 1982) and HMG proteins (Spiker, 1984). Gels were stained with Coomassie Blue R-250. Lane 1-purified wheat germ histone proteins; Lanes 2 and 3-NT-1 and tobacco leaf nuclei; Lane 4-GibcoBRL 10 kDa protein ladder; and Lane 5-purified wheat germ HMGs. Wheat histone and HMG protein bands are labeled. Tobacco H1 histones migrate to positions between the 40 and 50 kDa markers. The arrow indicates the band in the protein ladder corresponding to 30 kDa. The tobacco HMG I/Y homologs migrate to this position. (B) Western blot analysis of solubilized NT-1 and tobacco leaf nuclei. A duplicate gel to that described in (A) was blotted onto polyvinylidene fluoride membrane (Millipore) and probed with polyclonal antisera raised against wheat germ HMGa (Spiker and Everett, 1987). The CDP-star chemiluminescent detection system (Roche) was used to detect bound antibody according to manufacturer's directions. The positions of molecular mass standards are indicated on the left.

We have explored the hypothesis that differentiation state and HMG-I/Y content contribute to the great differences in MAR-related stimulation of transgene activity when cells in culture versus cells in intact plants are the objects of primary transformation. When cell culture lines were derived from transgenic plants, there was no preferential increase in GUS expression in MAR cell lines relative to leaf tissue. In addition, we did not observe differences in the level of the tobacco HMG-I/Y homolog between cells and leaf tissue.

We have shown that the state of differentiation cannot provide a simple explanation for the differences in MAR-mediated stimulation of transgene expression that we have previously observed when tobacco NT-1 cells and tobacco plants were the objects of the transformation event. There are several possible explanations for our results. It is possible that rate of cell division may be involved, as the SR-1 cell lines are dividing at approximately half the rate of the NT-1 cells. We consider this explanation unlikely, however, as both cell-culture types are dividing much more rapidly than the essentially quiescent cells in the plant leaves.

We consider two other explanations more likely. First, the greater MAR effect in NT-1 cells may be due to changes that have occurred over years in culture. Second, the state of cell differentiation at the time of transformation may be the operative variable. If the second possible explanation is true, MARs may exert their effects at the time of transformation by influencing the site of integration of transgenes into the recipient genome.

There is also evidence that the apparent MAR enhancement of gene expression is the result of reducing the incidence of gene silencing (Ülker et al., 1999; Vain et al., 1999; Ascenzi, unpublished observations). If this is the case, then the divergent effects of MARs that we have observed may be the result of differences in the ability of each cell type to bring about gene silencing. For example, differences in methylation potential may also come into play at the time of transformation, and the methylation machinery may be especially active in NT-1 cultures. Higher global methylation levels, as reported for cells undergoing antibiotic selection in cell culture (Schmitt et al., 1997), would potentially increase the probability that any given locus will become silenced. If MARs prevent the spread of methylation-dependent silencing, as reported by Forrester and colleagues (1999), then one might expect that the MAR effect would be more pronounced in an environment with higher methylation potential. In this case, one would expect to observe a larger proportion of low expressing lines containing the control construct as compared with the MAR lines.

In conclusion, we observed no preferential transgene expression enhancement in MAR lines relative to control lines upon conversion from differentiated cells to rapidly-dividing cells in culture. We also find, in contrast to observations in mammalian systems, that differences in the magnitude of enhancement of transgene expression in MAR lines do not correlate with the differences between HMG-I/Y levels in cells in culture and fully differentiated tissues in plants.

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